AN ULTRASTRUCTURAL STUDY OF α- AND β- CONIDIA IN THE FUNGAL GENUS *PHOMOPSIS*

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ABSTRACT. – In the genus *Phomopsis*, germination of the α-conidia has been demonstrated in many species, whereas the nature and function of β-conidia have been the subject of controversy due to their failure to germinate. Irregular enlargements along *P. helianthi* β-conidia were observed in 2-3 days in artificial culture, but in most cases these enlarged conidia disintegrated; during a 4-year study it was only exceptionally that the mycelium threads which could be seen by day 5 underwent further development.

Marked differences were observed between mature α- and β-conidia with regard to cellular components. An isodiametric nucleus occupying a central position in the cytoplasm; mitochondria with numerous, long cristae; large, numerous lipid droplets situated at the poles; polysaccharide and protein reserves in the form of granule rosettes, were recognised in α-conidia.

The presence of an elongate nucleus, a few mitochondria with a small number of cristae, lipid but not polysaccharide and protein reserves, was evidenced for the first time in β-conidia. The relatively early exhaustion of lipid reserves and the lack of other storage material available could account, at least in part, for the rapid senescence of β-conidia.

RÉSUMÉ. – Dans le genre *Phomopsis* la germination des conidies α a été démontrée chez plusieurs espèces, tandis que la nature et la fonction des conidies β a fait l'objet de controverses en raison de leur incapacité de germer. Chez *P. helianthi* des grossissements irréguliers tout au long des conidies β ont été observés au bout de 2-3 jours en culture artificielle, mais dans la plupart des cas ces conidies β élargies se sont désintégrées; au cours d'une étude menée pendant 4 ans ce ne fut qu'exceptionnellement que les filaments mycéliens qu'on pouvait voir au 5e jour ont continué à se développer.

Des différences marquantes ont été observées entre les conidies mûres α et β en ce qui concerne leurs organites. Un noyau isodiamétrique occupant une position centrale dans le cytoplasme, des mitochondries avec de nombreuses et longues crêtes, de grosses et nombreuses gouttelettes lipidiques situées vers les pôles, des réserves polysaccharidiques et protéiques en forme de rosette, ont été identifiés chez les conidies α.

La présence d'un noyau allongé, des mitochondries en petit nombre et à crêtes peu nombreuses, des réserves en lipides mais pas en polysaccharides et protéines, a été mise en évidence pour la première fois chez les conidies β. L'épuisement relativement rapide des réserves lipidiques et le manque d'autres substances de réserve pourraient expliquer, au moins en partie, la sénescence prématurée des conidies β.

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A prominent characteristic of the genus *Phomopsis* (Sphaeropsidales) is the production of two types of conidia : one fusoid-shaped, and the other filiform. Species presenting both these conidial types were originally considered as a section of the very large genus *Phoma* Sacc. In 1905 this section was definitely split off and described as a new genus, *Phomopsis* Sacc., whose history has been reviewed by HAHN (1930).

The two types of conidia were designated by DIEDICKE (1911) as α- and β-, but they have also been termed macroconidia, and microconidia or stylospores, respectively (WEHMEYER, 1975). The ability of α-conidia to germinate is not contested; in contrast, the nature and function of β-conidia have been the subject of controversy. SACCARDO (1905) considered these filiform structures as separate basidia (*basidia filiformis*), whereas TRAVERSO (1905) regarded them as released sporophores. Von HÖHNEL (1906), instead, referred to them as definite «stylospores», such as TULASNE, NITSCHKE, and FUCKEL had done previously. Later, GROVE (1917) and other investigators considered these structures as true spores, but a few authors used the term «free paraphyses» (REDDICK, 1909; FAWCET,1912; CAYLEY, 1923).

In the key for the Sphaeropsidales presented by Von ARX (1974) the genus *Phomopsis* was ultimately characterized by the presence of 1-celled conidia, with «filamentous pseudoconidia often also present», the word «pseudoconidia» indicating the questionable nature of these structures. SUTTON (1973), on the other hand, employed the term «conidia» for both α- and β-types.

Considering the divergence of opinion as to whether the β-spores are typical spores or only paraphyses or «basides», CAYLEY (1923) pointed out that «Repeated attempts to germinate these β-spores have failed, and ... they do not appear to have any definite nucleus». GROVE (1935), however, referring to *P. arctii* Trav., stated that «the β-spores will germinate». More recently, based upon results obtained using Giemsa, staining and light-microscope observations, VAN JAARSVELS & KNOX-DAVIES (1974) stated that conidiophores and stylospores were both uninucleate, and that in contrast to nuclei of α-conidia and of β-conidiophores, the stylospores nuclei were poorly resolved. According to these authors, stylospores failed to germinate after a week at room temperature on Water Agar, Potato Dextrose Agar, or Lupin Stem Agar.

Different accounts are found in the literature with regard to the presence of only one or both types of conidia in the same species, formed in the same versus in separate pycnidia. Seasonal changes, weather conditions (HARTER, 1917; HERR & al., 1983), nutrients and other factors have been considered important in the variation of α- and β-conidia proportions. HARTER & FIELD (1913) for the anamorph of *Diaporthe batatatis* Harter & Field, and PEZET (1974) for *P. viticola* (Sacc.) Sacc. reported that media which favoured stylospore

production were rich in carbohydrates. On the other hand, WELCH & GILMAN (1948) indicated that in *D. phaseolorum* var. *sojae* (Lehman) Wehmeyer «the production of the beta type was irregular, and attempts to associate its production with specific cultures or with a specific growth phase failed». This contradicts the assertion of WEHMEYER (1975) that «the first formed are the «beta» conidia».

The extensive presence of β-conidia and the paucity of α-type in *P. helianthi* Munt.-Cvet. et al. (MUNTAÑOLA-CVETKOVIĆ & al, 1981; PETROV & al., 1981), as well as the survival of pycnidia containing exclusively β-conidia from one year to the next (MIHALJCEVIĆ & al., *in press*), raised an intriguing question as to the significance of these β structures and their role in the etiology of the disease.

The present work was undertaken with the following aims : 1) to determine, at the cultural level, the extension of the statement (MUNTAÑOLA-CVETKOVIĆ & al., 1981) that β-conidia are capable of germination; 2) to observe, at the light microscope level, the morphologic changes occurring in α- and β-conidia under routine laboratory conditions for germination; 3) to elucidate, at the Transmission Electron Microscope (TEM) level, the presence of nuclei and other cellular components in β-conidia; 4) to compare α- and β-conidia ultrastructure.

MATERIALS AND METHODS

For comparative data concerning α and β-conidia, cultures of different *Phomopsis* species were employed in this study since it was principally aimed to obtain a better knowledge of *P. helianthi* and this species produced exclusively or almost exclusively β-conidia during a 4-year study.

Cultures of *P. sojae* Lehm. isolated from soybeans and from overwintered sunflower debris in Voivodina (MUNTAÑOLA-CVETKOVIĆ & al., *in press*), as well as other *Phomopsis* species producing abundant α-conidia, were employed in the studies of this conidial type.

For β-conidia studies, *P. helianthi* conidiomata were obtained from : 1) freshly collected diseased sunflower stems; 2) 1-year old herbarium sunflower stems (Sept. 1983 - Sept. 1984) placed in wet chamber for 24 h; 3) overwintered sunflower stems placed on Malt Agar (MA), Oatmeal Agar (OA), or Potato Dextrose Agar (PDA), all of them from Voivodina.

Techniques for studies on α- and β-conidia germination.

Conidiomata formed on pure culture were allowed to reach full maturity, at which time conidia freely oozed out from the interior of the cavity and mucilaginous drops were observed at the surface of the ostioles. The surface of the exudate was gently touched with a capillary micropipette; up to 0.5 ml of

sterile water (pH 7.4) was immediately absorbed using the same pipette which contained the conidia, and serial dilutions were made until a concentration of 10^4/ml was obtained. The dilute spore suspension was disposed in hanging drops, or was poured over 1 mm thick, filtrated, 0.8 % MA or PDA layers placed on sterilized slides and covered with sterilized cover slips. The spores on each slide were counted (100-500). The slides were kept in wet microchambers at room light and temperature (25 ± 1°C) conditions and examined every 3 hours.

Electron microscopy. Exudate from mature conidiomata was collected and embedded in small agar blocks (3 mm diam.) for electron microscope studies. Sections of mature conidiomata were also studied. Fixation in 3 % glutaraldehyde was followed by postfixation in 2 % OsO_4, dehydration in a graded ethanol series and embedding in Araldite. Thin sections were collected on copper grids and contrasted in uranyl acetate and lead citrate. For polysaccharide localization thin sections were collected on golden grids and treated according to Thiéry's method with thiocarbohydrazide and silver proteinate (THIÉRY, 1967). Extraction was carried out on golden grids after the removal of osmium from ultra-thin sections with hydrogen peroxyde (KNIGHT & LEWIS, 1977). The grids were incubated on pronase (*Streptomyces griseus*) at 37°C for two hours, and finally, contrasted with uranyl-acetate and lead citrate. Thin sections were then examined using a Siemens Elmiscope 101 Electron Microscope.

RESULTS

1. – The α-conidia of *P. sojae* :

Morphology. Exclusively α-conidia were found in the *P. sojae* material under study. The conidia were obtained from pycnidia formed on 10 days old PDA colonies and were unicellular, oblong-fusoid in shape, (4.4)-6.6-(8.0) x (1.5)-2.0-(3.4) μm.

Germination. *P. sojae* α-conidia began to show morphologic changes after 3 h, at which time they became swollen and attained dimensions of (5.9)-7.0-(8.9) x (1.9)-2.3-(3.6) μm. Incipient germ tubes of 2.2 μm in length developed in 6 h in 10 % of the conidia. In 9 h ca. 100 % of the conidia had germinated and mycelial threads of up to 23.7 μm developed (Fig. 1-3). Further elongation and profuse ramification were common events which were somewhat influenced by change in technique. Germination was slower and the germ tubes thinner in water hanging drops as compared to those grown on nutrient agar films. Conidia in direct contact with the air germinated more rapidly than those which were more deeply immersed in water.

Ultrastructure. A round nucleus occupied a central position in the cytoplasm of α-conidia. Chromatin material was barely visible, and the nucleus appeared relatively homogenous. In the material routinely stained for electron microscopy

the nuclear envelope and mitochondria were poorly resolved, although the fixation and staining procedures were carried out carefully (Fig. 5). Large, numerous lipid droplets constituted the principal storage material of α-conidia and were usually situated at the poles. Small vacuoles with extremely electron-dense contents conglomerated closely around the lipid droplets. Extraction with the enzyme pronase made the small vacuoles electron-transparent, and their contents were digested (Fig. 4). This demonstrated that the vacuoles contained protein and thus suggested that protein reserves were present in this type of conidia.

The reaction product obtained in the cytochemical test for polysaccharides was localized to the cell wall, plasma membrane and cytoplasmic polysaccharide reserves. Small plasma membrane invaginations were sporadically found and were characterized by a heavy silver deposit. Mitochondria with long cristae were easily recognized on the micrographs.

The cell wall appeared to be double-layered, with no superficial ornamentation. A very fine, granular reaction product was also demonstrated on the membranes surrounding the lipid droplets (Fig. 6).

2. – The β-conidia of *P. helianthi* :

Morphology. The dimensions of *P. helianthi* β-conidia were constant under all the conditions studied, with an average of 26.5 x 1.3 μm, and lower extreme of 20.7 x 1.1 μm. Minor variations were noted only for the maximal dimensions: 29.6 μm from pycnidia formed on OA colonies, and 32.6 x 1.4 μm from pycnidia on sunflower stems placed on MA. Extreme lengths of 37.0 μm and 41.4 μm were measured in β-conidia from conidiomata formed in *P. helianthi* colonies on PDA and MA, respectively, when an *Actinomyces* sp. extract was placed in walls contiguous to the colonies.

Germination. Incipient morphologic changes characterized by irregular enlargements along the conidia were observed within 2 days in approximately 30 % of the conidia. In most cases the enlarged conidia disintegrated, but some elongated and formed mycelial threads which could be seen by the 5th day. Only in exceptional cases did these threads develop and produce normal colonies.

Ultrastructure. Conidia within the pycnidial cavity showed electron-dense cytoplasm and relatively closely packed organelles in the narrow cellular lumen (Fig. 13, 14). An elongate nucleus was observed in the longitudinal section (Fig. 14, 19). In some sections two or three nuclear structures were present, but these might have been portions of a single, elongate nucleus (Fig. 13). The chromatin material was located close to the nuclear envelope, forming a thin layer of electron-dense material (Fig. 15). In the cytoplasm, only a few mitochondria could be seen and a small number of cristae recognized (Fig. 13, 14). Membrane accumulations were regularly present in the conidia, usually as a plasma membrane invagination, projecting into the cytoplasm (Fig. 13, 14).

Membrane accumulations were conspicuous in β-conidia from the pycnidial exudate. Concentric membranes were found in the vacuoles (Fig. 17, 18), and various types of plasma membrane invaginations resembling lomasomes were recognized (Fig. 16, 20). A progressive loss of electron-density of the cytoplasm had taken place in some β-conidia together with the increase of membrane accumulations (Fig. 20, 21), as compared with conidia still inside the pycnidia.

Polysaccharide reaction products were localized to the cell wall, plasma membrane and concentric membranes (Fig. 17, 18). No reaction product in the form of reserve polysaccharides was found in the cytoplasm.

The storage material of β-conidia was poor. A large lipid droplet was usually located at one of the apices in the conidia inside the conidiomata, while smaller lipid droplets were found in the cytoplasm of conidia from the exudate (Fig. 13, 20). Differences were also found with respect to the cell wall, which was electron-transparent and hardly visible in the β-conidia from inside the pycnidia (Fig. 13, 14, 15) while in the conidia from the exudate the cell wall was easily seen, relatively thin, mono-layered, with a superficial ornamentation (Fig. 19-21). The cell wall and this ornamentation became very conspicuous when cytochemical staining techniques for polysaccharides were employed (Fig. 16, 17, 18).

DISCUSSION

Germination was a common process observed in α-conidia of many *Phomopsis* species studied in this laboratory and also reported by different authors. Instead, only short mycelial threads were occasionally produced by *P. helianthi* β-conidia, a fact which agrees with the reports of GÄRTEL (1972) concerning *P. viticola* and with those of other authors which have worked with *Phomopsis*. The few colonies obtained from *P. helianthi* β-conidia during a 4-years study must be considered exceptional and do not substantiate their etiology as spreaders of disease like other summer anamorphs.

When comparing the ultrastructure of α- and β-conidia, the following noteworthy differences were observed : 1) the numerous long cristae present in mitochondria of α-conidia, in contrast with the small number of cristae in β-conidium mitochondria; 2) the presence of polysaccharide and protein reserves, in the form of granule rosettes and in vacuoles, respectively, in α-conidia, all of which were absent in β-conidia.

On the basis of numerous biochemical studies (YAMAGUCHI & al., 1974; COULTER & ARONSON, 1977; BECKER, 1978; FONTANA & KRISHMAN, 1978; LENDERMANN & RAST, 1978), as well as ultrastructural and cytochemical investigations (MÜLLER & HOHL, 1975; WATTERS & al., 1975; BRAÑA & al., 1980; BULLOCK & al., 1980) it has been proven that glycogen is the major intracellular polysaccharide of many fungi. The polysaccharide nature of the granular reserves found in the cytoplasm of α-conidia of *P. sojae* has been demonstrated in the present study; these reserves are supposed to be constituted of glycogen granules.

The distribution of «dense body vesicles» during oospore maturation was investigated in *Saprolegnia furcata* and *S. ferax* (BEAKES & GAY, 1978; BEAKES, 1980). In fine structure studies two types of membrane bound storage inclusions were distinguished in sclerotia of *Sclerotinia minor* : polyphosphate bodies and protein bodies (BULLOCK & al., 1980). The extraction with pronase, applied in the present study, demonstrate the presence of proteins in the small vacuoles found in *P. sojae* α-conidia.

Certain differences were observed in cytoplasmic organization between β-conidia inside the conidiomata and those from the exudate. These were presumed to be related to the degree of conidial maturation. Differences in dimension and number of lipid droplets suggest that lipid reserves are mobilized and utilized during conidium exit from the pycnidial cavity. Evidence of lipid utilisation during fungal spore germination is provided by numerous electron microscope observations on the disappearance of lipid globules. Quantitatively, the most important lipids of many fungi are triacyl-glycerides which are usually regarded as storage materials (WEETE, 1981).

In triacylglycerides of *Lipomyces lipofer* changes in fatty acid composition occurs depending on culture age. The exhaustion of lipid reserves prior to conidial germination had not been previously reported. The relatively early exhaustion of lipid reserves and the lack or other storage materials might be one of the reasons for the rapid senescence of β-conidia in *P. helianthi*.

Differences in electron-density of the cell wall may reflect a change in cell wall composition during the maturation period, although these differences could also be caused by better penetration of fixatives through the exudate than through the pycnidial cavity.

In *P. helianthi* β-conidia membrane accumulations occupied a large portion of the cell lumen. These accumulations were closely connected to the plasma membrane from which they may have arisen. The membrane accumulations in *P. helianthi* β-conidia resembled lomasomes. The functional relationship of the membranous type of locamoses with plasma membrane and wall production has been suggested by WEISBERG & TURIAN (1971, 1974) in *Aspergillus nidulans* hyphae and conidia. Cytochemical reactivity of plasma membrane and plasmalemmasomes to polysaccharides was found in *A. flavus* hyphae (BOJOVIĆ-CVETIĆ & VUJIČIĆ, 1980). The polysaccharide contents of the accumulated membranes in β-conidia, detected by heavy deposits of silver grains, confirmed the morphologic continuity of the accumulated membranes with the plasma membrane. Although the invaginations are prominent features of the plasma membrane of most fungal spored (HESS, 1981), the accumulation of membranes together with the loss of density of the cytoplasm and the gradual disintegration of the cytoplasm suggest that the incapacity of β-conidia to germinate might be due to premature senescence. These cytologic features observed in β-conidia correspond to the changes observed during early phases of autolysis (TRINCI & RIGHELATO, 1970).

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LÉGENDES DES ILLUSTRATIONS

Fig. 1-3. *P. sojae* α-conidia after 6h, 9h, and 12h respectively, on PDA film at 25°C.

Fig. 1-3. Conidies α de *P. sojae* placées pendant 6h, 9h, et 12h, respectivement, sur un film à l'agar de pomme de terre à 25°C.

Fig. 4. Thin section of an α-conidium after digestion with pronase. The contents of small vacuoles (v) are nearly completely extracted.

Fig. 4. Coupe fine d'une conidie α après digestion avec de la pronase. Le contenu des petites vacuoles (v) a été presque totalement extrait.

Fig. 5. Routinely stained thin section of an α-conidium showing a nucleus (N), large lipid droplets (L), and small vacuoles (v) with electron-dense contents.

Fig. 5. Coupe fine colorée d'une conidie α présentant un noyau (N), de grosses gouttelettes de lipides (L), et de petites vacuoles (v) avec un contenu dense aux électrons.

Fig. 6. Cytochemical localization of polysaccharides in α-conidia. The reaction product is evident at the cell wall (CW), plasma membrane (PM) and its invaginations (PMI), as well as at the granular reserve polysaccharides (PS). Nucleus (N), mitochondria (M) with long cristae and lipid droplets (L) with a slight reaction product on the membrane are also visible.

Fig. 6. Localisation cytochimique des polysaccharides chez les conidies α. Le produit de la réaction est évident dans la paroi cellulaire (CW), la membrane plasmique (PM) et ses invaginations (PMI), ainsi que dans les aggégats granulaires des polysaccharides de réserve (PS). Un noyau (N), des mitochondries (M) avec de longues crêtes et des gouttelettes lipidiques (L) avec un produit de réaction faible dans la membrane sont aussi visibles.

Fig. 7-9. *P. helianthi* β-conidia after 24h on a MA film at 25°C (in vivo).

Fig. 7-9. Conidies β de *P. helianthi* au bout de 24h après avoir été mises à germer sur un film de malt gélosé à 25°C (in vivo).

Fig. 10. The same as Fig. 7-9, after 3 days (in vivo).

Fig. 10. Le même que dans les Fig. 7-9, après 3 jours (in vivo).

Fig. 11. A mycelial thread produced by a *P. helianthi* β-conidium (in vivo).

Fig. 11. Filament mycélien produit par une conidie β de *P. helianthi* (in vivo).

Fig. 12. A *P. helianthi* β-conidium on MA + extract of an *Actinomyces* sp. (strain 468) (in vivo).

Fig. 12. Une conidie β de *P. helianthi* sur malt gélosé + un extrait de l'*Actinomyces* sp. souche 468 (in vivo).

Fig. 13-15. *P. helianthi* β-conidia from inside a pycnidium. 13 : longitudinal section with two nuclear profiles (N), mitochondria (M), and plasma membrane invaginations (PMI); 14 : oblique section showing a long, narrow nucleus (N) and accumulation of concentric membranes; 15 : detail of Fig. 13, showing a nucleus with chromatin material located close to the nuclear envelope (NE).

Fig. 13-15. Conidies β de *P. helianthi* encore à l'intérieur de la pycnide. 13 : Section longitudinale avec deux profils de noyau (N), des mitochondries (M) et des invaginations de la membrane plasmique (PMI); 14 : Section oblique montrant un noyau long et étroit (N) et des accumulations de membranes concentriques; 15 : Détail de la Fig. 13 montrant un noyau avec du matériel chromatique localisé près de l'enveloppe nucléaire (NE).

Fig. 16. Thin section of a *P. helianthi* β-conidium from pycnidial exudate : the reaction product of the cytochemical test for polysaccharides is localized to the cell wall (CW), plasma membrane (PM) and its invaginations (PMI).

Fig. 16. Coupe fine d'une conidie β de *P. helianthi* obtenue à partir de l'exsudat sorti de la pycnide : le produit de réaction de l'essai cytochimique pour les polysaccharides se trouve localisé dans la paroi cellulaire (CW), la membrane plasmique (PM) et ▬ invaginations.

Fig. 17, 18. Thin sections of *P. helianthi* β-conidia from pycnidial exudate : the reaction product of the cytochemical test for polysaccharides is localized to the cell wall (CW), plasma membrane (PM), and to the concentric membranes in the vacuoles (V).

Fig. 17 et 18. Coupes fines des conidies β de *P. helianthi* obtenues de l'exsudat des pycnides : le produit de réaction de l'essai pour les polysaccharides ■ trouve localisé dans la paroi cellulaire (CW), la membrane plasmique (PM) et les membranes concentriques dans les vacuoles (V).

Fig. 19-21. Cytological aspects of β-conidia from pycnidial exudate : accumulated membrane elements and ■ decrease in cytoplasmic electron-density are evident.

Fig. 19-21. Aspects cytologiques des conidies β obtenues de l'exsudat des pycnides : des éléments membraneux accumulés et une diminution de densité aux électrons sont évidents dans le cytoplasme.